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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 15 October 2002 with an application for Letters Patent number 521984 made by THE HORTICULTURE AND FOOD RESEARCH INSTITUTE OF NEW ZEALAND LIMITED.

Dated 5 November 2003.

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NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

ENZYME AND POLYNUCLEOTIDES ENCODING SAME

We THE HORTICULTURE AND FOOD RESEARCH INSTITUTE OF NEW ZEALAND LIMITED, a New Zealand Company and Crown Research Institute (under the Crown Research Institutes Act 1992) having a place of business at Corporate office, Tennent Drive, Private Bag 11030, Palmerston North, New Zealand, , do hereby declare this invention to be described in the following statement:

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ENZYME AND POLYNUCLEOTIDES ENCODING SAME

Technical Field

The present invention relates to the enzyme *alpha*-farnesene synthase and to polynucleotide sequences encoding the enzyme. The invention also relates to nucleic acid constructs, vectors and host cells incorporating the polynucleotide sequences. It further relates to the production of *alpha*-farnesene and its use in products such as an insect attractant, a sex pheromone and other products. *Alpha*-farnesene may also be used to produce other products with characteristic aromas useful as flavours and fragrances.

Background Art

Alpha-farnesene (Figure 1) is an acyclic sesquiterpene hydrocarbon ($C_{15}H_{24}$; 3,7,11-trimethyl-1,3,6,10-dodecatetraene) that is either constitutively present or induced in a wide range of species.

The biosynthetic pathway for the sesquiterpenes branches off from the general terpenoid pathway, beginning with the allylic diphosphate ester farnesyl diphosphate (FDP, also shortened to FPP) (Bohlmann, *et al.*, Proc. Natl. Acad. Sci. U. S. A. 95, 4126-4133 (1998), Cane and Bowser, Bioorg. Med. Chem. Lett. 9, 1127-1132 (1999), Davis and Croteau, Top. Curr. Chem. 209, 53-95 (2000)). *Alpha*-farnesene is synthesised from FDP in a reaction that proceeds through a carbocation intermediate (Figure 2) and is catalysed by the sesquiterpene synthase *alpha*-farnesene synthase (Rupasinghe, *et al.*, J. Am. Soc. Hortic. Sci. 123, 882-886 (1998)). The pathway for sesquiterpene biosynthesis, the acetate/mevalonate pathway, is localised to the cytoplasm; in contrast to the pathways for monoterpene and diterpene biosynthesis, which occur in the chloroplast (Croteau, *et al.*, In Biochemistry and Molecular Biology of Plants, eds Buchanan, Gruissem and Jones, American Society of Plant Physiologists, 1250-1318 (2000); Lange, *et al.*, Proc. Natl. Acad. Sci. U. S. A. 97, 13172-13177 (2000)).

All known plant terpene synthases, however, whether monoterpene, sesquiterpene or diterpene, appear to be closely related. Similarities include the positioning of intron sequences (Trapp and Croteau, Genetics 158, 811-832 (2001)) and the presence of conserved sequences, such as an aspartate-rich DDXX(D,E) motif (Lesburg, *et al.*, Curr. Opin. Struct. Biol. 8, 695-703 (1998)). This motif is involved in the binding of metal ions, usually Mg^{2+}, that are necessary for catalysis. (Lesburg, *et al.*, Curr. Opin. Struct. Biol. 8, 695-703 (1998)).

Alpha-farnesene synthase has been partially purified from the skin of apple fruit (*Malus domestica* Delicious). However, poor recovery and instability of the partially purified enzyme restricted further purification (Rupasinghe, *et al.*, J. Am. Soc. Hortic. 125, 111-119 (2000)). To date the gene for *alpha*-farnesene synthase has not been isolated from any source.

Alpha-farnesene is an insect attractant. It is a sex pheromone in mice and insects. Oxygenated (including chemicals occurring on exposure to air) *alpha*-farnesene products (eg farnesol, farnesal) have characteristic aromas (flavour/fragrance use). Other uses for *alpha*-farnesene and its derivatives are as potent cancer prevention agents, and in plastic film synthesis.

There is also a link between both the levels of *alpha*-farnesene and its oxidation products and the development of superficial scald, a postharvest physiological disorder that appears as a dark coloration of the apple skin following cool storage (Watkins, *et al.*, Acta. Hort. 343, 155-160 (1993), Ju and Bramlage, J. Am. Soc. Hortic. Sci. 125, 498-504 (2000), Whitaker and Saftner, J. Agric. Food Chem. 48, 2040-2043 (2000), Rowan, *et al.*, J. Agric. Food Chem. 49, 2780-2787 (2001)). To date the causal relationship between *alpha*-farnesene and scald is still unclear (Ju and Curry, J. Am. Soc. Hortic. Sci. 125, 626-629 (2000), Rupasinghe, *et al.*, J. Am. Soc. Hortic. Sci. 125, 111-119 (2000)). Ethylene production and *alpha*-farnesene biosynthesis also appear to be closely associated (Watkins, *et al.*, Acta Hort. 343, 155-160 (1993), Fan, *et al.*, J. Agric. Food Chem. 47, 3063-3068 (1999)). Recently it has been shown that ethylene may regulate the biosynthesis of *alpha*-farnesene during fruit ripening by acting on the mevalonate pathway, specifically by inducing the conversion of hydroxymethylglutaryl CoA to mevalonic acid (Ju and Curry, J. Am.

Soc. Hortic. Sci. 125, 105-110 (2000), Ju and Curry, Postharvest Biol. Technol. 19, 9-16 (2000), Ju and Curry, J. Am. Soc. Hortic. Sci. 126, 491-495 (2001)).

It is an object of the invention to provide methods for *in vitro* synthesis of *alpha*-farnesene and/or for genetically modifying plants to alter the levels of *alpha*-farnesene synthase activities in plants.

Summary of the Invention

In a first aspect the invention provides an isolated polynucleotide encoding *alpha*-farnesene synthase.

In a further aspect the invention provides an isolated polynucleotide having the sequence shown in Figure 3 or a fragment or variant thereof encoding a polypeptide with *alpha*-farnesene synthase activity.

In a further aspect, the invention provides an isolated polynucleotide encoding the polypeptide shown in Figure 4 or encoding a variant or a fragment of that sequence which has *alpha*-farnesene synthase activity.

In a further aspect the invention provides an isolated *alpha*-farnesene synthase polypeptide.

In yet a further aspect, the invention provides an isolated *alpha*-farnesene synthase having the sequence shown in figure 4 or a fragment or variant thereof with *alpha*-farnesene synthase activity.

The polypeptides of the invention are useful for *in vitro* preparation of *alpha*-farnesene.

In a further aspect the invention provides a vector comprising a polynucleotide of the invention.

In yet a further aspect the invention provides a genetic construct comprising in the 5'-3' direction

(a) a promoter sequence; and

(b) an open reading frame polynucleotide encoding a polypeptide of the invention

Preferably the genetic construct also comprises a termination sequence.

In another aspect the invention provides a genetic construct comprising in the 5'-3' direction

(a) a promoter sequence; and

(b) a polynucleotide which hybridizes to a polynucleotide encoding a polypeptide of the invention

Preferably the genetic construct also comprises a termination sequence.

In a further aspect the invention provides a host cell comprising a genetic construct of the invention.

In still a further aspect, the invention provides a transgenic plant cell which includes a genetic construct of the invention.

In addition the invention provides a transgenic plant comprising such cells.

In another aspect the invention provides a method for preparing *alpha*-farnesene comprising the steps of

(a) culturing a cell which has been genetically modified with a polynucleotide of the invention to provide increased *alpha*-farnesene synthase activity;

(b) providing the cell with farnesyl diphosphate if necessary; and

(c) separating the *alpha*-farnesene produced.

This method of the invention allows use of biofermentation for a convenient method for preparing the product.

Brief Description of Drawings

The present invention will be better understood with reference to the accompanying drawings in which:

Figure 1 shows the structures of the isomers of *alpha*-farnesene.

Figure 2 shows the pathway for *alpha*-farnesene synthesis in apple.

Figure 3 shows the cDNA sequence that encodes *alpha*-farnesene synthase. The sequence was obtained from a cDNA library that was constructed from Royal Gala 150 days after full bloom (DAFB) apple skin.

Figure 4 shows the predicted amino acid sequence of *alpha*-farnesene synthase from apple skins. The DDXXD motif involved in the binding of the metal ions necessary for catalysis is in bold. The highly conserved consensus sequence (L, V)(V,L,A)(N,D)D(L,I,V)X(S,T)XXXE, also involved in metal ion binding, is underlined.

Figure 5 shows a GC-MS trace of headspace above Royal Gala apples showing (E,E) *alpha*-farnesene peak at retention time 42.57 minutes.

Figure 6 shows a GC-MS trace of headspace above Ni+ purified cell free extracts (in binding buffer) harbouring *alpha*-farnesene synthase cDNA showing (E,E) *alpha*-farnesene (retention time 43.09 minutes) and (Z,E) *alpha*-farnesene (retention time 42.29 minutes).

Detailed Description

In one embodiment of the invention, cells genetically modified to exhibit *alpha*-farnesene synthase activity are used for the production of *alpha*-farnesene. While the cells may potentially be of any cell type that can be grown in culture, it is currently preferred to use bacteria or yeast cells for producing *alpha*-farnesene (and its

oxidation products or derivatives). Preferred cells for use in the biofermentation processes of this embodiment are *E. coli, Lactobacillus* sp and other non-pathogenic bacteria or yeasts such as brewers yeast.

Alpha-farnesene (or derivatives of *alpha*-farnesene) produced by biofermentation may be used as pheromones for use in insect or rodent control; as flavour or fragrance additives to food, medicine, toothpastes or perfumes; for the manufacture of pharmaceuticals with anti-tumour, anti-candida, mucosal stabilizing, anti-inflammatory and anti-ulcerative properties ; for the manufacture of films and polymers for use in packaging and moulded articles, particularly degradable plastics, general agrochemical production, production of solvents for industrial cleaning (eg algaecides) and membranes for dewaxing solvents or oils.

In another aspect of the invention, the polynucleotides of the invention are used to prepare transgenic plants that over-express the *alpha*-farnesene synthase in at least some parts of the plant. In this way the invention is used to impart fragrance to flowers, repel or attract insects (either as indicator plants, host plants, or alternative hosts) or impart an altered flavour to fruit or prevent scald in fruit, or to extract pharmaceutical products or animal or insect efficacious extracts.

In one particular aspect the polynucleotides of the invention are used in plants of the order Rosaceae, particularly in the genus *Malus* to provide increased flavour in fruit..

In another aspect polynucleotides of the invention are used to decrease *alpha*-farnesene synthase activity in apple fruit. This may be achieved in several ways, for example by genetically modifying the apples so that *alpha*-farnesene synthase polynucleotide is transcribed in an antisense orientation which results in decreased *alpha*-farnesene synthase translation. Such fruit may then display decreased superficial scold in apple skin following cold storage or be less attractive to codling moth.

In another aspect the invention provides a method useful in apple breeding. Segments of the polynucleotide sequences of the invention may be used as probes to investigate the genetic makeup of candidate apple varieties with respect to *alpha*-farnesene

synthase activity. The presence of high levels of polynucleotides encoding *alpha*-farnesene synthase activity in the fruit of apples may be used to identify apples with added flavour and presence of low levels may be used to identify apples with favourable storage properties.

The amino acid sequence of one polypeptide, an *alpha*-farnesene synthase from apple, and that of the polynucleotide sequence encoding it are given in Figures 4 and 3 respectively. It will however be appreciated that the invention is not restricted only to the polynucleotide/polypeptide having the specific nucleotide/amino acid sequence given in Figures 3 and 4. Instead, the invention also extends to variants of the polynucleotide/polypeptide of Figures 3 and 4 which passes or encode *alpha* farnesene synthase activity.

The term "polynucleotide(s)" as used herein means a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases and includes DNA and corresponding RNA molecules, including hnRNA and mRNA molecules, both sense and anti-sense strands, and comprehends cDNA, genomic DNA and recombinant DNA, as well as wholly or partially synthesized polynucleotides. An hnRNA molecule contains introns and corresponds to a DNA molecule in a generally one-to-one manner. An mRNA molecule corresponds to an hnRNA and DNA molecule from which the introns have been excised. A polynucleotide may consist of an entire gene, or any portion thereof. Operable anti-sense polynucleotides may comprise a fragment of the corresponding polynucleotide, and the definition of "polynucleotide" therefore includes all such operable anti-sense fragments.

The term 'polypeptide(s)' as used herein includes peptides, polypeptides and proteins.

The phrase "variants with *alpha*-farnesene synthase activity" is used in recognition that it is possible to vary the amino acid/nucleotide sequence of a polypeptide/polynucleotide while retaining substantially equivalent functionality. The equivalent can be, for example, a fragment of the polypeptide, a fusion of the polypeptide with another polypeptide or carrier, or a fusion of a fragment with additional amino acids.

An "isolated" polypeptide is a polypeptide that has been identified and separated or recovered to be largely free of components of its natural environment, (that is so that the polypeptide comprises at least 50% of the polypeptides from its natural environment, preferably at least 80%, more preferably at least 90%). The term "isolated" polypeptide includes polypeptides in situ within recombinant cells. However generally isolated polypeptides will be prepared by at least one purification step.

An "isolated" polynucleotide is a nucleotide molecule that is identified and separated from at least one contaminant polynucleotide with which it is ordinarily associated.

Variant polynucleotide sequences also include equivalent sequences, which vary in size, composition, position and number of introns, as well as size and composition of untranslated terminal regions. Variant polynucleotides also include those encoding functionally equivalent polypeptides.

It will be understood that a variety of substitutions of amino acids is possible while preserving the structure responsible for activity of the polypeptides. Conservative substitutions are described in the patent literature, as for example, in United States Patent No 5,264,558 or 5,487,983. It is thus expected, for example, that interchange among non-polar aliphatic neutral amino acids, glycine, alanine, proline, valine and isoleucine, would be possible. Likewise, substitutions among the polar aliphatic neutral amino acids, serine, threonine, methionine, asparagine and glutamine could be made. Substitutions among the charged acidic amino acids, aspartic acid and glutamic acid, could probably be made, as could substitutions among the charged basic amino acids, lysine and arginine. Substitutions among the aromatic amino acids, including phenylalanine, histidine, tryptophan and tyrosine are also possible. Such substitutions and interchanges are well known to those skilled in the art.

Equally, nucleotide sequences encoding a particular product can vary significantly simply due to the degeneracy of the nucleic acid code.

A polynucleotide or polypeptide sequence may be aligned, and the percentage of identical nucleotides in a specified region may be determined against another

sequence, using computer algorithms that are publicly available. Two exemplary algorithms for aligning and identifying the similarity of polynucleotide sequences are the BLASTN and FASTA algorithms. The similarity of polypeptide sequences may be examined using the BLASTP algorithm. Both the BLASTN and BLASTP software are available on the NCBI anonymous FTP server (ftp://ncbi.nlm.nih.gov) under /blast/executables/. The BLASTN algorithm version 2.0.4 [Feb-24-1998], set to the default parameters described in the documentation of variants according to the present invention. The use of the BLAST family of algorithms, including BLASTN and BLASTP, is described at NCBI's website at URL http://www.ncbi.nlm.nih.gov/BLAST/newblast.html and in the publication of Altschul *et al.*, Nucleic Acids Res. 25, 3389-34023 (1997). The computer algorithm FASTA is available on the Internet at the ftp site ftp://ftp.virginia.edu/pub/fasta/. Version 2.0u4, February 1996, set to the default parameters described in the documentation and distributed with the algorithm, is also preferred for use in the determination of variants according to the present invention. The use of the FASTA algorithm is described in Pearson and LipmanProc. Natl. Acad. Sci. USA 85, 2444-2448 (1988), Pearson Methods in Enzymology 183,63-98 (1990).

The following running parameters are preferred for determination of alignments and similarities using BLASTN that contribute to E values (as discussed below) and percentage identity: Unix running command: blastall -p blastn -d embldb -e 10 -G 1 -E 1 -r 2 -v 50 -b 50 -I queryseq -o results; and parameter default values:

-p Program Name [String]

-d Database [String]

-e Expectation value (E) [Real]

-G Cost to open a gap (zero invokes default behaviour) [Integer]

-E Cost to extend a cap (zero invokes default behaviour) [Integer]

-r Reward for a nucleotide match (blastn only) [Integer]

-v Number of one-line descriptions (V) [Integer]

-b Number of alignments to show (B) [Integer]

-i Query File [File In]

-o BLAST report Output File [File Out] Optional

For BLASTP the following running parameters are preferred: blastall -p blastp -d swissprotdb -e 10 -G 1 -E 1 -v 50 -b 50 -I queryseq -o results

-p Program Name [String]

-d Database [String]

-e Expectation value (E) [Real]

-G Cost to open a gap (zero invokes default behaviour) [Integer]

-E Cost to extend a cap (zero invokes default behaviour) [Integer]

-v Number of one-line descriptions (v) [Integer]

-b Number of alignments to show (b) [Integer]

-i Query File [File In]

-o BLAST report Output File [File Out] Optional

The "hits" to one or more database sequences by a queried sequence produced by BLASTN, BLASTP, FASTA, or a similar algorithm, align and identify similar portions of sequences. The hits are arranged in order of the degree of similarity and the length of sequence overlap. Hits to a database sequence generally represent an overlap over only a fraction of the sequence length of the queried sequence.

The BLASTN and FASTA algorithms also produce "Expect" or E values for alignments. The E value indicates the number of hits one can "expect" to see over a certain number of contiguous sequences by chance when searching a database of a certain size. The Expect value is used as a significance threshold for determining whether the hit to a database, such as the preferred EMBL database, indicates true similarity. For example, an E value of 0.1 assigned to a hit is interpreted as meaning that in a database of the size of the EMBL database, one might expect to see 0.1 matches over the aligned portion of the sequence with a similar score simply by chance. By this criterion, the aligned and matched portions of the sequences then have a 90% probability of being the same. For sequences having an E value of 0.01 or less over aligned and matched portions, the probability of finding a match by chance in the EMBL database is 1% or less using the BLASTN or FASTA algorithm.

According to one embodiment, "variant" polynucleotides, with reference to each of the polynucleotides of the present invention, preferably comprise sequences having the same number or fewer nucleic acids than each of the polynucleotides of the present invention and producing an E value of 0.01 or less when compared to the polynucleotide of the present invention. That is, a variant polynucleotide is any

sequence that has at least a 99% probability of being the same as the polynucleotide of the present invention, measured as having an E value of 0.01 or less using the BLASTN or FASTA algorithms set at the parameters discussed above.

Variant polynucleotide sequences will generally hybridize to the recited polynucleotide sequence under stringent conditions. As used herein, "stringent conditions" refers to prewashing in a solution of 6X SSC, 0.2% SDS; hybridizing at 65°C, 6X SSC, 0.2% SDS overnight; followed by two washes of 30 minutes each in 1X SSC, 0.1% SDS at 65°C and two washes of 30 minutes each in 0.2X SSC, 0.1% SDS at 65°C. The variant polynucleotide sequences of the invention are at least 50 nucleotides in length.

Variant polynucleotides also include sequences which have a sequence identity of at least 60%, generally 70%, preferably 80%, more preferably 90%, even more preferably 95%, very preferably 98% and most preferably 99% or more to the nucleotide sequence given in the sequence listing herein.

In general, polypeptide sequences that code for the *alpha*-farnesene synthases of the invention will be at least 50%, generally at least 60%, preferably 70%, and even 80%, 85%, 90%, 95%, 98%, most preferably 99% homologous or more with the disclosed amino acid sequence. That is, the sequence similarity may range from 50% to 99% or more. In addition the invention includes polynucleotide sequences encoding these amino acid sequences.

Also encompassed by the invention are fragments of the polynucleotide and polypeptide sequences of the invention. Polynucleotide fragments may encode protein fragments which retain the biological activity of the native protein. Alternatively, fragments used as hybridisation probes generally do not encode biologically active sequences. Fragments of a polynucleotide may range from at least 15, 20, 30, 50, 100, 200, 400 or 1000 contiguous nucleotides up to the full length of the native polynucleotide sequences disclosed herein.

Fragments of the polypeptides of the invention will comprise at least 5, 10, 15, 30, 50, 75, 100, 150, 200, 400 or 500 contiguous amino acids, or up to the total number of amino acids in the full length polypeptides of the invention.

Variant is also intended to allow for rearrangement, shifting or swapping of one or more nucleotides or domains/motifs (from coding, non-coding or intron regions) from genes (including terpene synthases) from the same or other species, where such variants still provide a functionally equivalent protein or polypeptide of the invention or fragment thereof.

It is, of course, expressly contemplated that homologs to the specifically described *alpha*-farnesene synthase having the sequence of Figure 4 exist in other plants. Such homologs are also "variants" as the phrase is used herein.

A polynucleotide sequence of the invention may further comprise one or more additional sequences encoding one or more additional polypeptides, or fragments thereof, so as to encode a fusion protein. Systems for such recombinant expression include, but are not limited to, mammalian, bacteria and insect systems.

DNA sequences from plants other than *Malus domestica* which are homologs of the *alpha*-farnesene synthase of Figure 3 may be identified (by computer-aided database searching) and isolated following high throughput sequencing of cDNA libraries prepared from such plants. Alternatively, oligonucleotide probes based on the sequence of Figure 4 can be synthesized and used to identify positive clones in either cDNA or genomic DNA libraries from other plants by means of hybridization or PCR techniques. Probes should be at least about 10, preferably at least about 15 and most preferably at least about 20 nucleotides in length. Hybridization and PCR techniques suitable for use with such oligonucleotide probes are well known in the art. Positive clones may be analyzed by restriction enzyme digestion, DNA sequencing or the like.

The polynucleotides of the present invention may be generated by synthetic means using techniques well known in the art. Equipment for automated synthesis of oligonucleotides is commercially available from suppliers such as Perkin

Elmer/Applied Biosystems Division (Foster City, CA) and may be operated according to the manufacturer's instructions.

As a result of the identification of the polypeptides and polynucleotides of the invention alpha-farnesene activity may be modulated in plants. Modulation may involve a reduction in the expression and/or activity (i.e. silencing) of the polypeptide.

Any conventional technique for effecting this can be employed. Intervention can occur post-transcriptionally or pre-transcriptionally. Further, intervention can be focused upon the gene itself or on regulatory elements associated with the gene and which have an effect on expression of the encoded polypeptide. "Regulatory elements" is used here in the widest possible sense and includes other genes which interact with the gene of interest.

Pre-transcription intervention can involve mutation of the gene itself or of its regulatory elements. Such mutations can be point mutations, frameshift mutations, insertion mutations or deletion mutations. These latter mutations include so called "knock-out" mutations in which expression of the gene is entirely ablated.

Examples of post-transcription interventions include co-suppression or anti-sense strategies, a dominant negative approach, or techniques which involve ribozymes to digest, or otherwise be lethal to, RNA post-transcription of the target gene.
Co-suppression can be effected in a manner similar to that discussed, for example, by Napoli *et al. Plant Cell 2, 279-290* (1990) and de Carvalho Niebel *et al. Plant Cell 7, 347-358* (1995). In some cases, it can involve over-expression of the gene of interest through use of a constitutive promoter. It can also involve transformation of a plant with a non-coding region of the gene, such as an intron from the gene or 5' or 3' untranslated region (UTR).

Anti-sense strategies involve expression or transcription of an expression/transcription product capable of interfering with translation of mRNA transcribed from the target gene. This will normally be through the expression/transcription product hybridising to and forming a duplex with the target mRNA.

The expression/transcription product can be a relatively small molecule and still be capable of disrupting mRNA translation. However, the same result is achieved by expressing the whole polynucleotide in an anti-sense orientation such that the RNA produced by transcription of the anti-sense oriented gene is complementary to all or part of the endogenous target mRNA.

Anti-sense strategies are described generally by Robinson-Benion *et al.* Methods in Enzymol 254, 363-375 (1995) and Kawasaki *et al.*, Artific. Organs 20, 836-845 (1996).

Genetic constructs designed for gene silencing may include an inverted repeat. An 'inverted repeat' is a sequence that is repeated where the second half of the repeat is in the complementary strand, e.g.,
5'-GATCTA.....TAGATC-3'
3'-CTAGAT.....ATCTAG-5'
The transcript formed may undergo complementary base pairing to form a hairpin structure provided there is a spacer of at least 3-5 bp between the repeated regions.

Another approach is to develop a small antisense RNA targeted to the transcript equivalent to an miRNA (Llave *et al.*, Science 297, 2053-2056 (2002) that could be used to target gene silencing.

The ribozyme approach to regulation of polypeptide expression involves inserting appropriate sequences or subsequences (eg. DNA or RNA) in ribozyme constructs (McIntyre Transgenic Res. 5 257-262 (1996)). Ribozymes are synthetic RNA molecules that comprise a hybridizing region complementary to two regions, each of which comprises at least 5 contiguous nucleotides of a mRNA molecule encoded by one of the inventive polynucleotides. Ribozymes possess highly specific endonuclease activity, which autocatalytically cleaves the mRNA.

Alternately, modulation may involve an increase in the expression and or activity of the polypeptide by over-expression of the polynucleotide, or by increasing the number of copies of the polynucleotide in the genome of the host.

To give effect to the above strategies, the invention also provides genetic constructs usually DNA constructs. The DNA constructs include the intended DNA (such as one or more copies of a polynucleotide sequence of the invention in a sense or anti-sense orientation or a polynucleotide encoding the appropriate ribozyme), a promoter sequence and a termination sequence (which control expression of the gene), operably linked to the DNA sequence to be transcribed. The promoter sequence is generally positioned at the 5' end of the DNA sequence to be transcribed, and is employed to initiate transcription of the DNA sequence. Promoter sequences are generally found in the 5' non-coding region of a gene but they may exist in introns (Luehrsen Mol. Gen. Genet 225, 81-93 (1991)) or in the coding region.

A variety of promoter sequences which may be usefully employed in the DNA constructs of the present invention are well known in the art. The promoter sequence, and also the termination sequence, may be endogenous to the target plant host or may be exogenous, provided the promoter and terminator are functional in the target host. For example, the promoter and termination sequences may be from other plant species, plant viruses, bacterial plasmids and the like. Preferably, promoter and termination sequences are those endogenously associated with the *alpha*-farnesene synthase genes.

Factors influencing the choice of promoter include the desired tissue specificity of the construct, and the timing of transcription and translation. For example, constitutive promoters, such as the 35S Cauliflower Mosaic Virus (CaMV 35S) promoter, will affect the transcription in all parts of the plant. Use of a tissue specific promoter will result in production of the desired sense or antisense RNA only in the tissue of interest. With DNA constructs employing inducible promoter sequences, the rate of RNA polymerase binding and initiation can be modulated by external stimuli, such as light, heat, anaerobic stress, alteration in nutrient conditions and the like. Temporally regulated promoters can be employed to effect modulation of the rate of RNA polymerase binding and initiation at a specific time during development of a transformed cell. Preferably, the original promoters from the gene in question, or promoters from a specific tissue-targeted gene in the organism to be transformed are used. Other examples of promoters which may be usefully employed in the present

invention include, mannopine synthase (mas), octopine synthase (ocs) and those reviewed by Chua *et al.* Science 244, 174-181 (1989).

The termination sequence, which is located 3' to the DNA sequence to be transcribed, may come from the same gene as the promoter sequence or may be from a different gene. Many termination sequences known in the art may be usefully employed in the present invention, such as the 3' end of the *Agrobacterium tumefaciens* nopaline synthase gene. However, preferred termination sequences are those from the original gene or from the target species to be transformed.

The DNA constructs of the present invention may also contain a selection marker that is effective in cells, to allow for the detection of transformed cells containing the construct. Such markers, which are well known in the art, typically confer resistance to one or more toxins. One example of such a marker is the NPTII gene whose expression results in resistance to kanamycin or hygromycin, antibiotics which are usually toxic to plant cells at a moderate concentration. Alternatively, the presence of the desired construct in transformed cells can be determined by means of other techniques well known in the art, such as PCR or Southern blots.

Techniques for operatively linking the components of the inventive DNA constructs are well known in the art and include the use of synthetic linkers containing one or more restriction endonuclease sites. The DNA construct may be linked to a vector capable of replication in at least one system, for example, *E. coli*, whereby after each manipulation the resulting construct can be sequenced and the correctness of the manipulation determined.

The DNA constructs of the present invention may be used to transform a variety of plants including agricultural, ornamental and horticultural plants. In a preferred embodiment, the DNA constructs are employed to transform apple, banana, kiwifruit, tomato, cotton, rose, olive and potato plants.

As discussed above, transformation of a plant with a DNA construct including an open reading frame comprising a polynucleotide sequence of the invention wherein the open reading frame is orientated in a sense direction can, in some cases, lead to a

decrease in expression of the polypeptide by co-suppression. Transformation of the plant with a DNA construct comprising an open reading frame or a non-coding (untranslated) region of a gene in an anti-sense orientation will lead to a decrease in the expression of the polypeptide in the transformed plant.

It will also be appreciated that transformation of other non-plant hosts is feasible, including well known prokaryotic and eukaryotic cells such as bacteria (e.g. *E. coli*, *Agrobacterium*), fungi, insect, and animal cells is anticipated. This would enable production of recombinant polypeptides of the invention or variants thereof. The use of cell free systems (e.g. Roche Rapid Translation System) for production of recombinant proteins is also anticipated (Zubay Annu Rev Genet 7, 267-287 (1973)).

The polypeptides of the invention produced in any such hosts may be isolated and purified from same using well known techniques. The polypeptides may be used in cell-free systems for enzymic synthesis of *alpha*-farnesene.

Techniques for stably incorporating DNA constructs into the genome of target plants are well known in the art and include *Agrobacterium tumefaciens* mediated introduction, electroporation, protoplast fusion, injection into reproductive organs, injection into immature embryos, high velocity projectile introduction, floral dipping and the like. The choice of technique will depend upon the target plant to be transformed.

Once the cells are transformed, cells having the DNA construct incorporated into their genome may be selected by means of a marker, such as the kanamycin resistance marker discussed above. Transgenic cells may then be cultured in an appropriate medium to regenerate whole plants, using techniques well known in the art. In the case of protoplasts, the cell wall is allowed to reform under appropriate osmotic conditions. In the case of seeds or embryos, an appropriate germination or callus initiation medium is employed. For explants, an appropriate regeneration medium is used.

In addition to methods described above, several methods are well known in the art for transferring DNA constructs into a wide variety of plant species, including gymnosperms angiosperms, monocots and dicots.

The resulting transformed plants may be reproduced sexually or asexually, using methods well known in the art, to give successive generations of transgenic plants.

The nucleotide sequence information provided herein will also be useful in programs for identifying nucleic acid variants from, for example, other organisms or tissues, particularly plants, and for pre-selecting plants with mutations in *alpha*-farnesene synthase or their equivalents which renders those plants useful in an accelerated breeding program to produce plants in which the content of *alpha*-farnesene and its derivatives is modulated. More particularly, the nucleotide sequence information provided herein may be used to design probes and primers for probing or amplification of *alpha*-farnesene synthase. An oligonucleotide for use in probing or PCR may be about 30 or fewer nucleotides in length. Generally, specific primers are upwards of 14 nucleotides in length. For optimum specificity and cost effectiveness, primers of 16-24 nucleotides in length are preferred. Those skilled in the art are well versed in the design of primers for use in processes such as PCR.

If required, probing can be done with entire restriction fragments of the gene disclosed herein. Naturally, sequences based upon Figure 4 or the complements thereof can be used. Such probes and primers also form aspects of the present invention.

Methods to find variants of the of polynucleotides of the invention from any species, using the sequence information provided by the invention, include but are not limited to, screening of cDNA libraries, RT-PCR, screening of genomic libraries and computer aided searching of EST, cDNA and genomic databases. Such methods are well known to those skilled in the art.

The invention will now be illustrated with reference to the following non-limiting Example.

EXAMPLE

The following Example further illustrates practice of the invention.

Plant material and GC-MS analysis: Tree-ripened 150 DAFB apples (*Malus domestica*) were harvested from Royal Gala trees grown in a HortResearch orchard at Hawkes Bay, New Zealand. Twelve fruit were selected for analysis and were placed into a 5 L wide-necked round-bottomed sampling vessel with a ground glass flat flange joint. The vessel was covered with a glass lid with a sealed ground glass joint inlet socket containing a gas line and a volatile sorbent cartridge containing 100 mg Chromosorb 105. The headspace in the flask was allowed to equilibrate at 23°C for 1 hour, after which the headspace was purged with N_2(g) at 25.0 ml/min while being trapped for 15 min. The Chromosorb cartridge was dried with a N_2(g) flow at 10 psi, 35 C for 15 min prior to analysis. The volatiles were thermally desorbed from the Chromosorb traps for 3 min at 150°C into the injection port of the gas chromatograph (GC) HP5890. The GC system was equipped with a DB-Wax capillary column (J & W Scientific, Folsom, USA), 30 m x 0.32 mm i.d., with a 0.5 μm film thickness. The carrier gas was helium at a flow rate of 30 cm/sec. The GC oven was programmed to remain at 30°C for 6 min, then to increase by 3°C/min to 102°C, followed by an increase of 5°C/min to 190°C, which was maintained for 5 min. The column outlet was split to a mass spectrometer (VG70SE), in addition to the GC's flame ionisation detector (GC-FID/MS). The mass spectrometer operated in electron impact ionisation (EI-MS) mode at 70 eV with a scan range 30 – 320 amu. Component identification was assisted with mass spectra of authentic standards, library spectra (NIST and in-house) and GC retention indices. Quantitative data was obtained by measuring the sample peak areas relative to an authentic standard.

Isolation of mRNA and cDNA library construction: The skin of the 150 DAFB apples was removed with a peeler and total RNA was extracted from the peeled skin by an adaptation of the method of Gomez and Gomez (Langenkamper, *et al.*, Plant Mol. Biol. 36, 857-869 (1998)). mRNA purified from the total RNA by oligo(dT)-cellulose chromatography (Pharmacia) was used to construct a Lambda ZAP-CMV (Stratagene) cDNA library according to the manufacturer's instructions. The cDNA-

containing pBK-CMV plasmids were massed excised and used to transform *E. coli* XLOLR (Stratagene). The plasmids were isolated from the XLOLR colonies and partially sequenced. All sequences on the database were BLASTed against the NRBD90 database (Altschul, *et al.*, Nucleic Acids Res. 25, 3389-3402 (1997).) and putative terpene synthase cDNA sequences were identified by their similarity to known terpene synthases based on key motifs. A full-length terpene synthase sequence (EST57400) was identified and its polynucleotide sequence determined.

Cloning into pET30: For functional expression, a cDNA fragment encoding EST57400 was excised from pBK-CMV57400 using a *Eco*RI restriction endonuclease site immediately adjacent to the start ATG and the vector *Xho*I restriction site. The resultant 1899 bp cDNA sequence was then subcloned in frame into the expression vector pET30a (Novagen), which was also digested with *Eco*RI and *Xho*I, yielding plasmid pET30a57400. Plasmid pET30a57400 was then transformed into *E. coli* BL21-CodonPlus™-RIL cells (Stratagene). The clone was resequenced at the 5' end to ensure the inserted cDNA was in frame.

Expression and characterization of *alpha*-farnesene synthase from bacterial cultures: *E. coli* BL21-Plus™-RIL cells harbouring pET30a57400, and empty pET30 vector as a control, were grown overnight at 37°C in Lauria-Bertani media supplemented with 30 μg/ml kanamycin and 50 μg/ml chloramphenicol. A 500 μl aliquot of overnight culture was used to inoculate 50 ml of fresh 2 x YT medium supplemented with 30 μg/ml kanamycin and 50 μg/ml chloramphenicol. The culture was grown at 37°C with vigorous agitation to A_{600} = 0.6 before induction with 0.3 mM isopropyl-ß-D-thiogalactopyranoside (IPTG) and simultaneous addition of farnesyl diphosphate (FDP) (10 μM). The culture was immediately transferred to a 30°C incubator, or 16 or 37°C incubators depending on the experiment.

Headspace analysis of bacterial cultures: The headspace in the vessels above the bacterial cultures was collected immediately after the addition of FDP using solid phase micro extraction (SPME). The SPME fibres (65 μm PDMS/DVB, Supelco, Australia) were conditioned for 45 min at 260 °C and the background analysed for contamination using GC-FID (HP5890) prior to use. The headspace volatiles were collected for 4 hours at 30°C with continuous agitation (110 rpm). Prior to analysis

using a GC-FID/MS, the fibres were stored at ambient temperature in septum sealed glass vials. The volatiles were desorbed from the fibres for 5 minutes at 250°C in the GC injection port. The GC system was equipped with a DB-Wax capillary column (J & W Scientific, Folsom, USA), 30 m x 0.25 mm i.d., with a 0.5 μm film thickness. The carrier gas was helium at a flow rate of 30 cm/sec. The GC oven was programmed to remain at 30°C for 6 min, then to increase by 3°C/min to 102°C, followed by an increase of 5°C/min to 210°C, which was maintained for 11 min. The mass spectrometer operated in electron impact ionisation (EI-MS) mode at 70 eV with a scan range of 30 – 320 amu. Peak identification was carried out by comparison of sample spectra with those from NIST, Wiley, and our own mass spectra libraries and confirmed by retention indices of authentic standards and literature values (Davies, J. Chrom. 503, 1-24, (1990)). Quantitative data was obtained by measuring sample peak area relative to an internal standard, hexadecane, which had been added to the cultures at the same time as the FDP.

Expression time course for induced and non-induced cultures: 6 x 50 ml bacterial cultures harbouring pET30a57400 were prepared as above. At A_{600} = 0.6 three of the cultures were induced with 0.3mM IPTG leaving the remaining cultures non-induced. Cultures were then incubated for one, three or five hours at 30°C and the headspace volatiles were collected as described above.

Characterization of *alpha*-farnesene synthase from bacterial extracts and partially purified *alpha*-farnesene synthase recombinant protein: Cultures were set up, grown and induced as above. Following induction, cultures were immediately transferred to a 24°C incubator and allowed to grow for a further 18-20 hours with continuous agitation and then cells harvested by centrifugation (2000 x g for 10min). Pelleted cells were resuspended in either 20 ml binding buffer (5 mM imidazole, 0.5 mM NaCl, 10 mM DTT, 20 mM Tris-HCl (pH 7.9) or 20 ml extraction buffer (25 mM MOPS (pH 7.0), 10 mM sodium ascorbate, 25 mM KCl, 10 mM DTT, 10% glycerol). Cells were disrupted with 2 x exposure to 12,700 psi in a French Pressure Cell Press (American Instrument Co. Inc, Silver Spring, Maryland. USA) and then centrifuged at 8000 x g for 15min. 5 ml of supernatant was transferred to a 50 ml test-tube and adjusted to 10 mM $MgCl_2$ and 20 μM $MnCl_2$. FDP (100 μM) was added

and the reaction mixture was incubated at 30°C. Headspace volatiles were collected as in the same manner as whole cultures. The remainder of the extract (15 ml) was applied to PD-10 gel filtration columns (Amersham-Pharmacia Biotech) pre-equilibrated with either binding or extraction buffer (DTT omitted). Eluent fractions were then pooled and purification of recombinant protein was carried out in a single step using immobilised metal affinity chromatography (IMAC). The eluent was applied to a Hi–Trap Chelating HP column (Amersham-Pharmacia Biotech) charged with Ni^{+}. Non bound proteins were removed and recombinant protein was eluted following the manufacturer's specifications. Five ml samples of the eluted protein were transferred to 50 ml test-tubes and adjusted to 10 mM $MgCl_2$, 20 μM $MnCl_2$ and 10 μM FDP was added. Headspace volatiles were collected as in the bacterial cultures. Aliquots of the remaining recombinant protein were stored at -80°C in 20% glycerol until required.

Electrophoresis and Western analysis: Whole culture, French Press His-purified and non His-purified protein extracts were analysed by SDS-PAGE, using 10% polyacrylamide gels. Protein bands were either visualised using Colloidal Coomassie or were transferred on to Immobilin-P PVDF membrane (Millipore). Blotted proteins were incubated with Anti-His_6 monoclonal (Roche) primary and Anti-Mouse IgG-AP (Stressgen) secondary antibodies and were detected using 1-STEP™ NBT/BCIP (Pierce) alkaline phosphatase detection reagent.

Protein quantification: Protein concentrations of extracts and partially purified recombinant proteins were determined according to Bradford using the Biorad kit according to manufacturers specification using a Spectromax Plus spectrophotometer, using bovine serum albumin (BSA) as the standard.

Results

Headspace analysis of volatiles emitted from 150 DAFB apples: It is well established that *alpha*-farnesene is synthesised in apple skin tissue and detected in headspace analyses. Typically two isomers of *alpha*-farnesene are found in apple skin, (E,E) and (Z,E) *alpha*-farnesene (Matich, *et al.*, Anal. Chem. 68, 4114-4118

(1996), Bengtsson, *et al.*, J. Agric. Food Chem. 49, 3736-3741 (2001)). These two isomers are usually identified in the ratio of 100:1 respectively (Matich, *et al.*, Anal. Chem. 68, 4114-4118 (1996)). In the headspace of the 150 DAFB apples analysed only the 'all trans' (E,E) isomer of *alpha*-farnesene was identified (Figure 5). This isomer was present at low levels, on average 4 ng (E,E) *alpha*-farnesene per fruit. The (E,E) *alpha*-farnesene isomer had a retention time of 42.57 minutes that was used to calculate the Kovats retention index for this compound. The retention index and the mass spectra positively identified this compound as (E,E) *alpha*-farnesene.

Sequence analysis of *alpha*-farnesene synthase: Sequencing of the cDNA in pBK-CMV that encoded *alpha*-farnesene synthase revealed an insert size of 1926 base pairs excluding the poly(A) tail (Figure 4). The cDNA sequence had a predicted ORF of 576 amino acids beginning with a putative start methionine 61 bases in from the 5' end (Figure 3). The molecular mass of *alpha*-farnesene synthase is predicted to be 66kD. The predicted amino acid sequence of *alpha*-farnesene synthase does not have a chloroplast-signalling peptide sequence (Emanuelsson, *et al.*, 300, 1005-1016 (2000)), which is typical of monoterpene and diterpene synthases. As has been found for all other terpene synthases the predicted amino acid sequence of *alpha*-farnesene synthase contains a DDXX(D,E) motif (DDVYD) at amino acids 326 to 330 that is involved in the binding of the metal ions necessary for catalysis. Another sequence, LNNDLGTSAAE, that corresponds to the highly conserved consensus sequence (L,V)(V,L,A)(N,D)D(L,I,V)X(S,T)XXXE, which is also involved in metal ion binding (Rynkiewicz, *et al.*, Proc. Natl. Acad. Sci. U. S. A. 98, 13543-13548 (2001)), is located at amino acids 469 to 479.

Bohlman et al (Proc. Natl. Acad. Sci. U. S. A. 95, 4126-4133 (1998)) made a comparison between the amino acid sequences of 28 terpene synthases. They showed that there were 22 absolutely conserved amino acid residues and four highly conserved amino acid residues. An examination of the amino acid sequence of *alpha*-farnesene synthase shows that 19 of the 22 absolutely conserved amino acids and three of the four highly conserved amino acids are present. They also found that six positions were absolutely conserved for aromatic amino acids and four positions were absolutely conserved for acidic amino acids. In *alpha*-farnesene synthase, four of the six aromatic positions and all of the four acidic positions are conserved. They then

extended the analysis to include all 33 terpene synthases that were known at the time and showed that there were seven absolutely conserved amino acid residues. *Alpha*-farnesene synthase contains six of these seven absolutely conserved amino acids. The predicted isoelectric point is 5.2. This is similar to the isoelectric point calculated for other sesquiterpene synthases. For example, two sesquiterpene synthases isolated from *Artemisia annua*, cASC34 and cASC125, have isoelectric points of 5.28 and 5.50, respectively (Van Geldre, *et al.*, Plant Sci. 158, 163-171 (2000)).

Surprisingly, the predicted amino acid sequence for *alpha*-farnesene synthase (a sesquiterpene synthase) most closely resembles the amino acid sequences of monoterpene synthases. *Alpha*-farnesene synthase is most similar to a putative chloroplastic monoterpene synthase from *Quercus ilex* (holly oak) (Fischbach, Genbank, 2001), having 41% identity and 60% similarity from predicted amino acids 33 to 575. An isoprene synthase precursor from *Populus x canescens* (poplar) has the second highest similarity (Miller, *et al.*, Planta 213, 483-487 (2001)), with 40% identity and 57% similarity from predicted amino acids 33 to 573, and limonene synthase from *Perilla citriodora* has the third highest similarity (Ito, *et al.*, Biol. Pharmacol. Bull. 23, 359-362 (2000)), with 39% identity and 58% similarity from predicted amino acids 31 to 572. The first sesquiterpene synthase that *alpha*-farnesene synthase most closely resembles is a sesquiterpene cyclase from *Capsicum annuum* (Bell pepper) that is involved in capsidiol formation (Back, *et al.*, Plant Cell Physiol. 39, 899-904 (1998)), having 32% identity and 54% similarity from predicted amino acids 33 to 570. The nucleic acid sequence of the *alpha*-farnesene synthase shows homology to very short stretches of the mRNA of a few sesquiterpene synthases. One area of homology lies between nucleotides 918 and 946. For example, cadinene synthase from *Gossypium arboreum* (tree cotton) (Chen, *et al.*, J. Nat. Prod. 59, 944-951 (1996)) has 24 out 25 bases identical in the region between nucleotides 918 and 946 and a putative sesquiterpene synthase from *Artemisia annua* (sweet wormwood) has 25 out 26 bases identical in this region (Van Geldre, *et al.*, Plant Sci. 158, 163-171 (2000)). Between nucleotides 367 and 386, (E) α-bisabolene synthase of *Abies grandis* (grand fir) (Bohlmann, *et al.*, Proc. Natl. Acad. Sci. U. S. A. 95, 6756-6761 (1998)) has 20 out of 20 bases identical.

The cDNA sequence for *alpha*-farnesene synthase (EST 57400) was obtained from a cDNA library constructed from Royal Gala 150 DAFB apple skin. Three other truncated cDNAs with polynucleotides across the sequenced 5' end identical to EST 57400 were also isolated. One was from Royal Gala 126 DAFB fruit cortex, one from Royal Gala floral buds, and the third from Pinkie leaf. Another truncated cDNA obtained from Aotea leaf had seven base pair differences out the 675 bases sequenced.

Western analysis: Western analysis confirmed the presence of a soluble expression product within the expected size range (80-85kDa His tag inclusive) for *alpha*-farnesene synthase in both the French Press extracts and partially purified recombinant protein extracts. No similar-sized band was detected in either the pET30a control purified or non-purified extracts.

Characterisation of *alpha*-farnesene synthase (E,E)-*alpha*-farnesene and small amounts (Z,E)-*alpha*-farnesene were detected in the headspace of bacterial cultures and extracts harbouring pET30a57400. Controls comprising *E. coli* BL21 cells transformed with pET30a lacking the *alpha*-farnesene synthase cDNA insert gave negligible or no *alpha*-farnesene. (E,E)-*alpha*-farnesene production in both cultures and crude extracts, although not dependent on precursor addition, was shown to be dependent on the presence of the *alpha*-farnesene synthase cDNA insert. Although a peak was found at a similar retention time in the control as the *alpha*-farnesene (42.24 min), the mass spectra showed this to be citral. Addition of GDP to bacterial cultures did not produce either *alpha*-farnesene or any monoterpenes.

Headspace analysis of partially purified recombinant enzyme, whether derived from extraction in His purification binding buffer or sesquiterpene extraction buffer, showed (E,E)-*alpha*-farnesene as the major product with minor amounts of (Z,E)-*alpha*-farnesene present (Fig 6). This required added FDP, no *alpha*-farnesene was produced without the added percursor. Purified enzyme that had been stored in glycerol for 4 weeks at –80 C was reassayed with only 15% loss of activity.

The bacterial cultures and crude extracts harbouring the *alpha*-farnesene cDNA showed leaky expression under non-inducing conditions. However volatile trapping

over a 5 hour period demonstrated that addition of IPTG increased the production of both isomers relative to the samples that were not induced.

EST 57400 therefore encodes an *alpha*-farnesene synthase that makes only *alpha*-farnesene.

The above Example is an illustration of practice of the invention. It will be appreciated by those skilled in the art that the invention can be carried out with numerous modifications and variations. For example, variations to the nucleotide sequences may be used and the sequences may be expressed in different organisms.

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By the authorised agents
A J PARK
Per

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15 OCT 2002
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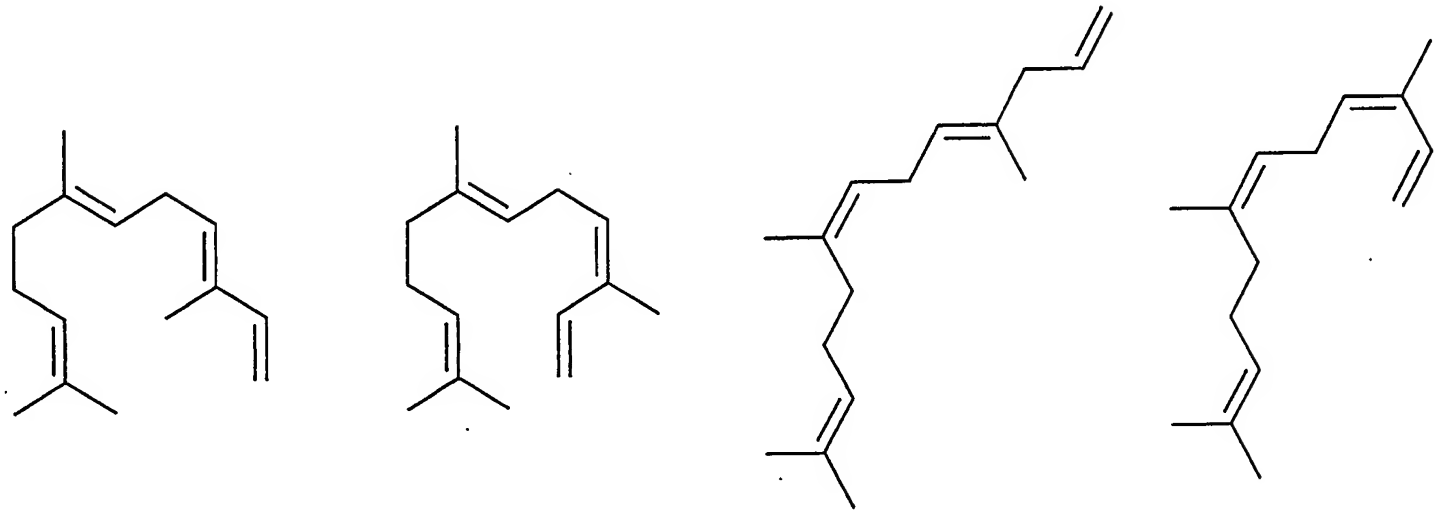
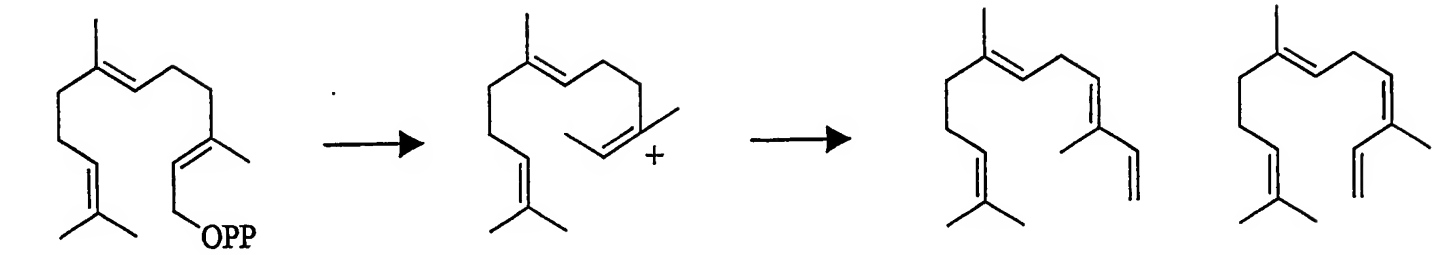


(E,E) α-farnesene (Z,E) α-farnesene (E,Z) α-farnesene (Z,Z) α-farnesene

FIGURE 1



Farnesyl diphosphate Farnesyl carbocation (E,E) α-Farnesene (Z,E) α-Farnesene

FIGURE 2


1 CTATAGCTTC TTGTATCCCA AACATCTCGA GCTTCTTGTA CACCAAATTA GGTATTCACT ATCGAATTCA GACTTCACTT GCAAGCTGAT AATGAGCAGA
101 AAATTTTTCA AAACCAGATG AAACCCGAAC CTGAAGCCTC TTACTTGATT AATCAAAGAC GGTCTCGAAA TTACAAGCCA AATATTTCGA AGAACGATTT
201 CCTAGATCAA TCTCTTATCA GCAAATACGA TCGAGATGAG TATCGGAAGC TGTCTGAGAA GTTAATAGAA GAAGTTAAGA TTTATATATC TGCTGAAACA
301 ATGGATTTAG TAGCTAAGTT CGAGCTCATT GACAGCGTCC GAAAACTAGG CCTCGCGAAC CTCTTCGAAA AGGAAATCAA CGAAGCCCTA GACAGCATTG
401 CAGCTATCGA AAGCGACAAT CTCGGCACAA GAGACGATCT CTATCGTACT GCATTACACT TCAACATCCT CAGGCAGCAT GGCTATAAAG TTTCACAAGA
501 TATATTTGGT AGATTCATCG ATGAAAAGCG CACATTAGAG AACCACCATT TCCCCCATTT AAAAGGAATG CTCGAACTTT TCGAGGCCTC AAACCTGGGT
601 TTCGAAGGTG AAGATATTTT AGATGAGGCG AAAGCTTCCT TGACGCTAGC TCTCAGAGAT AGTGGTCATA TTTGTTATCC AGACAGTAAC CTTTCCAGGG
701 ACGTAGTTCA TTCCCTGGAG CTTCCATCAC ACCGCAGAGT GCAGTGGTTT GATGTCAAAT GGCAAATCAA CGCCTATGAA AAAGACATTT GTCGCGTCAA
801 CGCCACGTTA CTCGAATTAG CAAAGCTTAA TTTCAACGTA GTTCAGCCCC AACTCCAAAA AAACTTAAGG GAAGCATCCA GGTGGTGGGC AAATCTGGGC
901 TTCGCAGACA ACTTGAAATT TCCAAGAGAT AGACTGGTTG AATGTTTCTC ATGTGCTGTG GGAGTAGCAT TCGAGCCTGA GCACTCATCT TTTAGAATAT
1001 GTCTTACCAA AGTCATCAAC TTAGTACTGA TCATAGACGA CGTCTATGAT ATTTATGGCT CAGAGGAAGA GCTAAAGCAC TTCACCAATG CTGTTGATAG
1101 GTGGGATTCT ACGGAAACTG ACGAGCTTCC AGAGTGTATG AAGATGTGTT TCCAAGTACT CTACAACACT ACTTGTGAAA TTGCTCGTGA AATTGAGGAG
1201 GAGAATCGTT CGAACCAAGT ATTACCTCAA TTGACCAAAG TGTCGGCAGA TTTTTGTAAA GCATTATTGG TGGAGGCAGA GTGGTATAAT AAGAGCCATA
1301 TACCAACCCT TGAAGAGTAC CTAAGAAACG GATGCATTTC ATCATCAGTT TCAGTGCTTT TGGTTCAGTC GTTTTTCTCT ATAACTCATG AGGGAACCAA
1401 AGAGATGGCT GATTTTCTTC ACAAGAATCA AGATCTTTTG TATAATATCT CTCTCATCGT TCGCCTCAAC AATGATTTCG GAACTTCCGC GGCTGAACAA
1501 GAGAGACGGG ATTCTCCTTC ATCAATCGTA TGTTACATGA GAGAAGTGAA TGCCTCTGAA GAAACAGCTA CGAAGAACAT TAAGGGCATG ATAGACAATG
1601 CATCGAAGAA AGTAAATGGA AAATGCTTCA CAACAAACCA AGTGCCTTTT CTGTCATCAT TCATGAACAA TGCCACAAAC ATGGCACGTG TGGCGCACAG
1701 CCTTTACAAA GATGGAGATG CGTTTCGTGA CCAAGAGAAA GGGCCTCGGA CCCACATCCT GTCTTTACTA TTCCAACCTC TTGTAAACTA GTACTCATAT
1801 AGTTTGAAAT AAATAGCAGC AAGAAGTTTG CGGTTCAGTT CGTCATGGAT AAATTAATCT TTACAGTTTG TAACGTTGTT GCACAAAGAT TATGAATAAA
1901 AAGTTGTAGT TTGTCGTTTA TTTTTTAAAA AAAAAAAAAA AAAAAAAAAA AA

FIGURE 3

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  1  MEFRVHLQAD NEQKIFQNQM KPEPEASYLI NQRRSANYKP NIWKNDFLDQ
 50  SLISKYDGDE YRKLSEKLIE EVKIYISAET MDLVAKLELI DSVRKLGLAN
100  LFEKEIKEAL DSIAAIESDN LGTRDDLYGT ALHFKILRQH GYKVSQDIFG
150  RFMDEKGTLE NHHFAHLKGM LELFEASNLG FEGEDILDEA KASLTLALRD
200  SGHICYPDSN LSRDVVHSLE LPSHRRVQWF DVKWQINAYE KDICRVNATL
250  LELAKLNFNV VQAQLQKNLR EASRWWANLG FADNLKFARD RLVECFSCAV
300  GVAFEPEHSS FRICLTKVIN LVLIIDDVYD IYGSEEELKH FTNAVDRWDS
350  RETEQLPECM KMCFQVLYNT TCEIAREIEE ENGWNQVLPQ LTKVWADFCK
400  ALLVEAEWYN KSHIPTLEEY LRNGCISSSV SVLLVHSFFS ITHEGTKEMA
450  DFLHKNEDLL YNISLIVRLN NDLGTSAAEQ ERGDSPSSIV CYMREVNASE
500  ETARKNIKGM IDNAWKKVNG KCFTTNQVPF LSSFMNNATN MARVAHSLYK
550  DGDGFGDQEK GPRTHILSLL FQPLVN*
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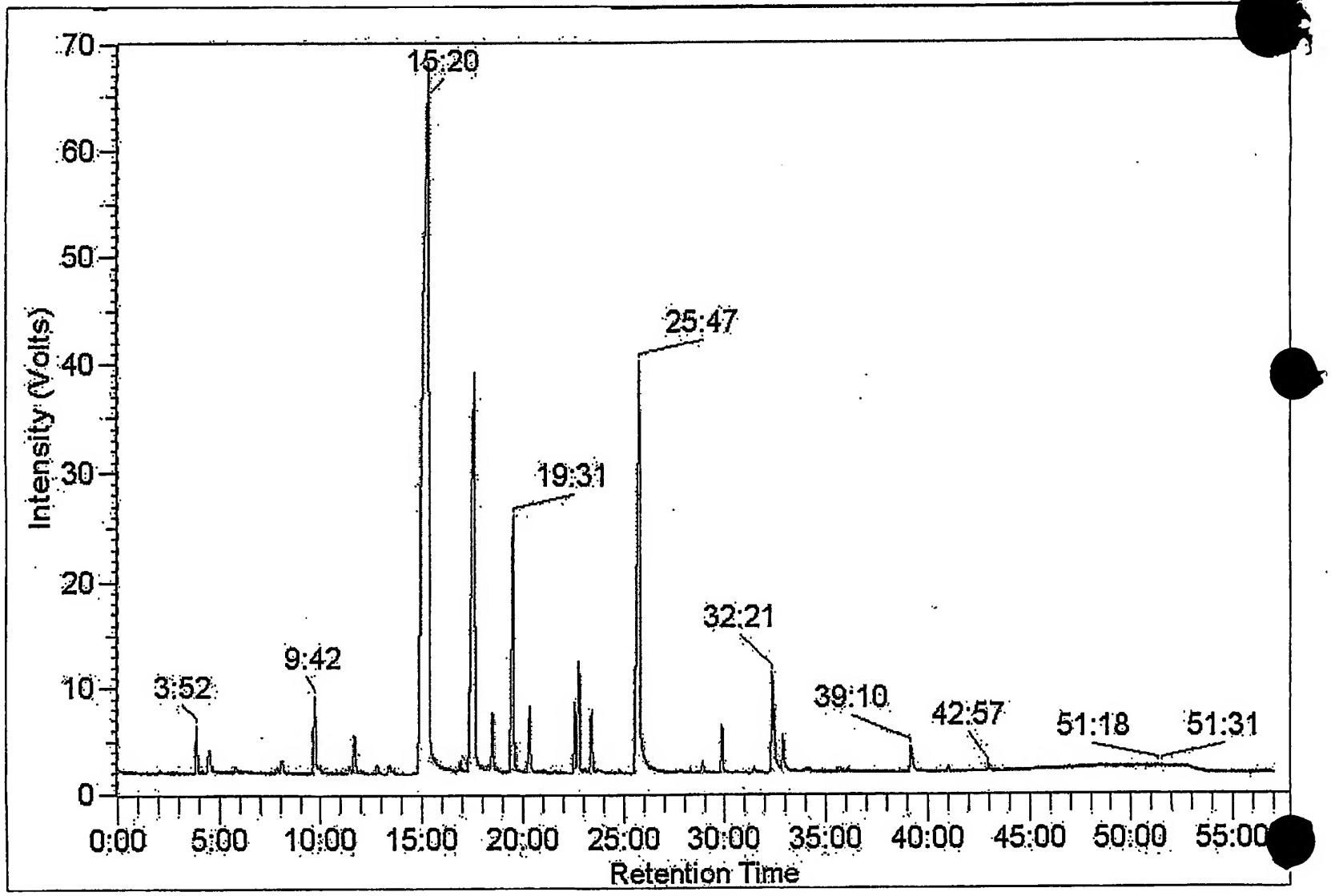
FIGURE 4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50
40
30
20
10
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19:31
32:21
9:42
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42:57
51:18
51:31
0:00
5:00
10:00
15:00
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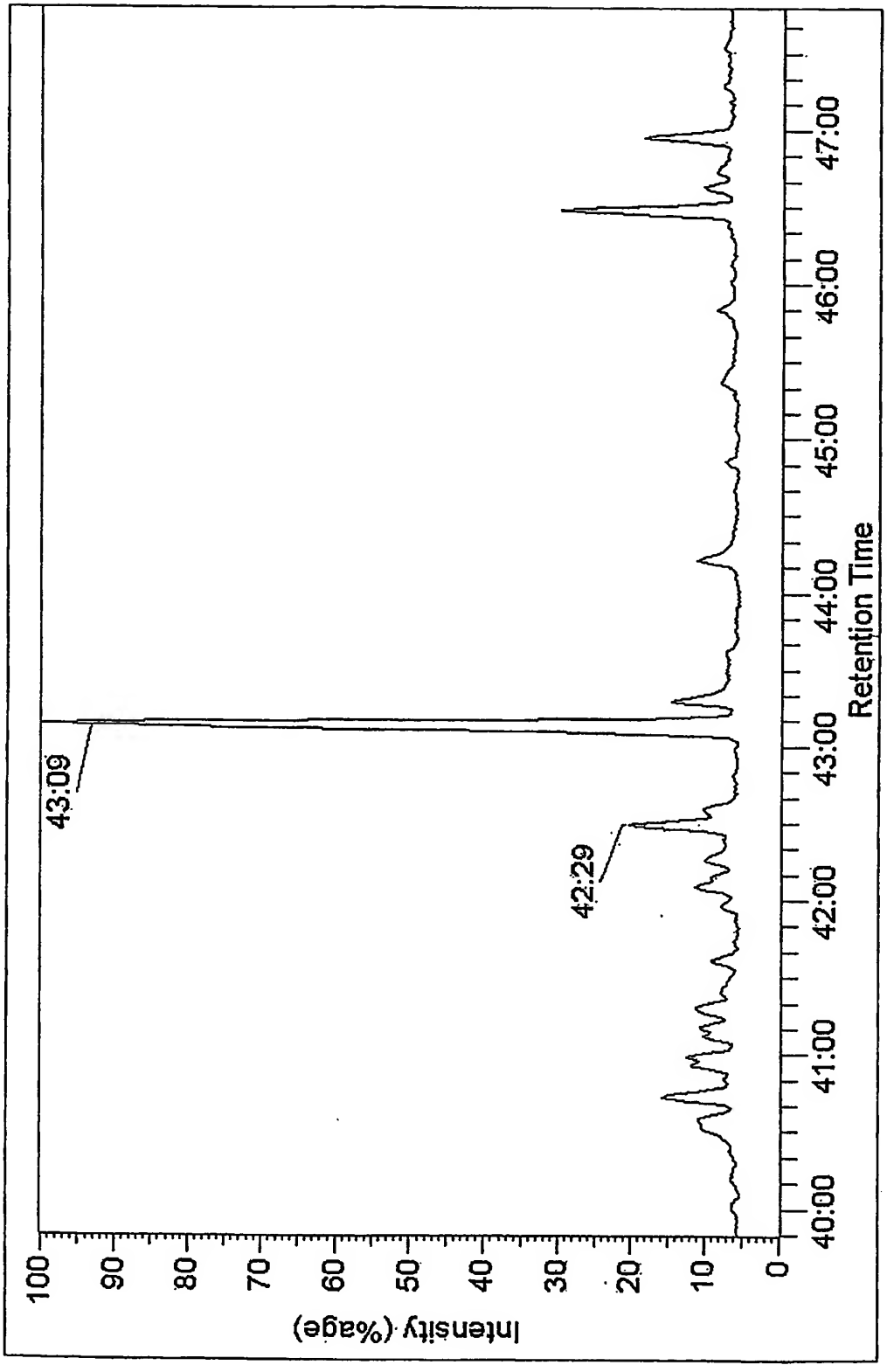
25:00
30:00
35:00
40:00
45:00
50:00
55:00
Retention Time

FIGURE 5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70
60
50
40
30
20
10
0
40:00
41:00
42:00
43:00
44:00
45:00
46:00
47:00
Retention Time
43:09
42:29

FIGURE 6